

# *Acanthamoeba* Keratitis in Non-Contact Lens Wearers in India

## DNA Typing–Based Validation and a Simple Detection Assay

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**Objectives:** To establish that the protozoan *Acanthamoeba* is one of the causative organisms associated with non-contact lens–related keratitis in the Indian population and to develop a simple and sensitive diagnostic assay for clinical testing.

**Design:** DNA sequencing of nuclear 18S and 26S ribosomal DNA motifs was performed and compared with the reference *Acanthamoeba* strains, to establish the genetic identity of the putative amoeba isolates obtained from the corneal scrapings of non-contact lens-wearing patients with keratitis. Ribosomal DNA typing of clinical corneal scrapings from the patients with keratitis was performed by means of a simple agarose gel–based multiplex polymerase chain reaction assay, to detect the cases of *Acanthamoeba* keratitis.

**Results:** The ribosomal DNA analysis of 15 putative amoeba isolates obtained from the corneal scrapings of 14 patients with keratitis and 1 from the patients' environment established the isolates to be pathogenic forms

of *Acanthamoeba* belonging to type T4 ribosomal DNA genotype. Multiplex polymerase chain reaction assay was specific and sensitive enough to detect as low as 5 pg of *Acanthamoeba* DNA. Its utility as a reliable diagnostic assay was demonstrated directly with the use of 34 additional corneal scrapings.

**Conclusions:** *Acanthamoeba* is one of the causative organisms of keratitis in Indian patients with no history of contact lens usage. Moreover, the *Acanthamoeba* infection can be easily detected in the clinical samples by means of the simple multiplex polymerase chain reaction assay based on ribosomal DNA typing.

**Clinical Relevance:** This study suggests the need and means to determine the incidence and prevalence of *Acanthamoeba* keratitis in India and elsewhere. Moreover, the polymerase chain reaction assay would help in early and definitive diagnosis, leading to better prognosis of *Acanthamoeba* keratitis condition.

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**A**CANTHAMOEBA KERATITIS has been described primarily in reports from developed countries, with several studies suggesting soft contact lens wear as the greatest risk factor.<sup>1-4</sup> In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers.<sup>5</sup> This apparent low incidence of *Acanthamoeba* keratitis in India has resulted from

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the belief that the disease is related mainly to contact lens wear—a factor usually absent in most cases of keratitis from this part of the world—and also from the unavailability of simple and sensitive diagnostic tools for its clinical detection. A consequence of this low reporting is the skepti-

cal acceptance of *Acanthamoeba* as a pathogenic organism by the medical community as well as the health authorities in India. Although our group<sup>5</sup> reported a number of cases, all of these were based on fluorescence microscopy of the corneal scrapings and culture on nonnutrient agar with *Escherichia coli* overlay. These techniques involving culture establishment and fluorescence microscopy require considerable experience and sophisticated infrastructure that are not available to most ophthalmologists, and thus data remain lacking from other parts of India. In comparison, direct detection methods based on routine light microscopy of smears and histologic preparations, although widely available to clinicians, are not only less sensitive but also unreliable. It has been estimated that 60% to 70% of cases of *Acanthamoeba* keratitis are misdiagnosed by such methods.<sup>6</sup> The situation thus calls for newer,

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cost-effective, sensitive, simple, and reliable diagnostic tools that can be easily integrated in a small to medium-sized clinical setup, leading to more realistic estimates of the disease incidence as well as helping early diagnosis and treatment of the disease for better response from the patients.

In this study, we provide DNA-based evidence that *Acanthamoeba* is associated with keratitis in Indian patients with no history of contact lens usage. In addition, we report a simple, rapid, and sensitive agarose gel-based multiplex polymerase chain reaction (PCR) assay for reliable detection of *Acanthamoeba* in clinical samples.

## METHODS

### SAMPLES USED FOR MOLECULAR VALIDATION

Twenty-two amoeba isolates obtained from the corneal scrapings of non-contact lens-wearing patients who had nonbacterial and nonfungal keratitis were used for DNA analysis-based genetic identity. These comprised 15 isolates from India (14 from patients with keratitis and 1 from potable water from the home of one of the patients), 1 each from Pakistan and Argentina, 4 American Type Culture Collection (ATCC) strains, and 1 standard strain of *Acanthamoeba culbertsoni*. Of the 14 keratitis samples from India, 12 (**Table**) were collected at L. V. Prasad Eye Institute, Hyderabad, and the other 2 were from Sankara Nethralaya, Chennai. All isolates were grown axenically as monolayers in PYG (protease peptone-yeast-glucose) medium.

### SAMPLES USED FOR DEVELOPMENT OF PCR-BASED DIAGNOSTIC ASSAY

Initial standardization of a PCR-based diagnostic assay was performed with the samples used for molecular validation described in the preceding paragraph. Subsequently, the assay was tested for its reliability and utility in clinical diagnosis directly on corneal scrapings from 34 patients diagnosed and treated at L. V. Prasad Eye Institute from January 1, 2000, through October 31, 2001. These samples were selected from among the 2213 cases of microbial keratitis with no history of contact lens usage. All the 34 corneal scraping samples were collected in duplicate, of which one set was stored in phosphate-buffered saline and the other set was directly investigated for the causative organism by our laboratory-specific protocol<sup>5</sup> that includes fluorescence microscopy. The latter investigation showed 25 corneal scrapings to be culture positive for *Acanthamoeba* and 9 for bacteria or fungus.

The clinical data and results of microbial investigations of all the amoeba-positive samples, except for those obtained from outside India, are given in the Table. The study was approved by the institutional ethics committee of L. V. Prasad Eye Institute.

### DNA ISOLATION

For ribosomal DNA (rDNA) analysis, total DNA was extracted from the amoebic cultures and corneal scrapings by means of the UNSET (urea-NaCl-SDS-EDTA-Tris hydrochloride) lysis buffer method.<sup>7</sup> To isolate DNA from cultures, amoebae were harvested from 4- to 5-day-old, 5-mL confluent cultures (approximately  $1 \times 10^6$  amoebae, containing approximately 9:1 ratio of trophozoites and cysts) by centrifugation at approximately 1000g for 5 minutes. The harvested cells were washed twice with 5 mL of phosphate-buffered saline and resuspended in 0.5 mL of UNSET lysis buffer for DNA isolation.

### Clinical Findings and Other Details of Amoeba-Positive Patients With Keratitis

Patient and Sample Details	Use of Samples	
	Molecular Validation* (n = 12)	Multiplex PCR (n = 25)
Sex, No. M/F	9/3	15/10
Age, y		
Mean $\pm$ SD	40.7 $\pm$ 14.4	34.4 $\pm$ 14.7
Range	25-63	7-63
Socioeconomic status, No.		
Very poor	7	18
Poor-middle class	5	7
Predisposing factors, No. (%)		
Contact lens wear	0	0
Injury	1 (8)	6 (24)
Foreign body	3 (25)	6 (24)
Unknown	8 (67)	13 (52)
Identification of amoebae, putatively <i>Acanthamoeba</i> , in corneal scrapings, No. (%)†		
Smear staining		
Calcofluor white	11 (92)	24 (96)
Gram stain	9 (75)	21 (84)
Giemsa	8 (67)	16 (64)
Culture on nonnutrient agar	12 (100)	24 (96)
Treatment outcome, No. (%)		
Healed with medical therapy‡	9 (75)	15 (60)
Surgical intervention		
Penetrating keratoplasty	1 (8)	1 (4)
Evisceration	0	2 (8)
Lost to follow-up, No. (%)	2 (17)	7 (28)

Abbreviation: PCR, polymerase chain reaction.

\*Data from 4 clinical isolates obtained from outside Hyderabad, India, are not included.

†For experimental details of staining and culture, see Sharma et al.<sup>5</sup>

‡Polyhexamethylene biguanide and chlorhexidine digluconate, each 0.02%.

The aqueous lysate was extracted twice with 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was finally precipitated with 0.1 vol of 3M sodium chloride and 2 vol of ethanol and dissolved in 200  $\mu$ L of  $1 \times$  TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH 8.0). For corneal scraping samples, a small amount of corneal tissue (approximately 0.2-0.5 mm) scraped by means of a surgical blade (No. 15 on Bard Parker handle) with the patient under topical anesthesia was collected in 1 mL of phosphate-buffered saline and stored at  $-20^\circ\text{C}$  until testing. For DNA isolation, the stored corneal scraping samples were pelleted, lysed in 0.5 mL of UNSET lysis buffer, and then processed as described in the preceding sentences, except that the organic-phase extraction was done only once and final DNA was dissolved in only 50  $\mu$ L of  $1 \times$  TE buffer. All centrifugations for DNA isolation were carried out at 8000g for 8 to 10 minutes in a centrifuge (Biofuge Fresco; Heraeus Instruments, Osterode, Germany).

### RIBOSOMAL DNA ANALYSIS

Each sample was used to amplify 5 genus- or pathotype-specific genomic regions based on the published literature, of which 2 targets specific to 18S rDNA<sup>8</sup> and 26S rDNA<sup>9</sup> giving 463-base pair (bp) and 126-bp fragments, respectively, were found to be most promising for characterization as well as diagnosis of *Acanthamoeba*. Primer sequences used to amplify 18S rDNA domain were as follows: 5'-GGCCCAGATCGTTTAC-

CGTGAA-3' and 5'-TCTCACAAGCTGCTAGGGGAGTCA-3' (oral communication, Thomas J. Byers, PhD, Ohio State University, Columbus), whereas those for 26S rDNA target were as follows: 5'-GGAGCTCCCACGGGAGGCC-3' and 5'-TGGACCGGTGAGGCTGCGGCT-3', as described by Lai et al.<sup>9</sup> Amplified products were sequenced on an automated DNA sequencer (ABI-3700; Applied Biosystems, Foster City, Calif) and aligned with those of reference sequences (identified by BLAST [basic local alignment search tool] search and retrieved from the EMBL [European Molecular Biology Laboratory] database) using ClustalX (available at: <http://www.igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>). The sequence data were then used to construct phylogenetic trees to infer the genetic identities of the amoeba isolates analyzed in the present study. Moreover, to ascertain the confidence values of the rDNA sequence-based inferences regarding the genetic identities, sequence data were subjected to bootstrap analysis.<sup>10</sup> For the latter purpose, the aligned sequences were resampled 100 times by means of SEQBOOT software and the resampled data set was then used to compute genetic distances (DNADIST) and consensus distance trees showing genetic relationships by means of unweighted pair group method using arithmetic averages followed by CONSENSE programs. All programs were run with the use of PHYLIP package 3.6, which also contains simplified documentation files of each of these sequence analysis programs used in the study (available at: <http://evolution.genetics.washington.edu/phylip.html>).

For rDNA typing of isolates as well as for diagnostic assay, the PCRs were done in 20- $\mu$ L reactions using 1 and 3  $\mu$ L of template DNA (for amoebae and corneal scraping samples, respectively). Each PCR contained 1 U of *Taq* DNA polymerase (AmpliTaq Gold; PerkinElmer, Inc, Boston, Mass), 2 $\mu$ M of each primer, 200 $\mu$ M dNTPs (deoxyribose nucleotide triphosphates), and 1.5mM magnesium chloride and was amplified for 35 three-step cycles of 94°C, 61°C, and 72°C, each for 1 minute, followed by final extension of 72°C for 5 minutes in an MJR PTC-200 thermocycler (MJ Research Inc, Waltham, Mass). In each PCR amplification, the cycling profile was preceded by one heating step of 94°C for 10 minutes to activate the *Taq* DNA polymerase. To develop a direct gel-based assay for *Acanthamoeba* detection in clinical samples, primers for the 2 rDNA targets were tested individually and also in combination, initially with DNA from culture-proved and "Type" isolates of *Acanthamoeba* and then with the use of DNA from 34 corneal scraping samples from patients with keratitis. The amplified products were resolved and visualized on ethidium bromide-stained 1.5% agarose gel. In all the experiments, proper negative (water in place of template DNA) and positive (DNA from *Acanthamoeba castellanii*, ATCC 50370) controls were used. Moreover, specificity of the assay was tested with DNA from 2 *Acanthamoeba*-related protozoa, *Balamuthia mandrillaris* and *Hartmannella vermiformis* (ATCC 30966), and human leukocytes, *Pseudomonas aeruginosa*, *Aspergillus* species, and herpes simplex virus. The sensitivity was determined by testing 10-fold dilutions of *A castellanii* DNA.

## RESULTS

All the rDNA sequences specific to 22 amoeba isolates analyzed in the study were deposited in GenBank (accession numbers AF-534135 to AF-534179). The rDNA-based phenogram (Figure 1) showed all the Indian isolates, including the environmental one, and those from Argentina and Pakistan, to be *Acanthamoeba* having type T4 sequence, the type found most commonly associated with the pathogenic isolates causing keratitis in contact lens wearers.<sup>7,8,11</sup> The genetic identities were supported

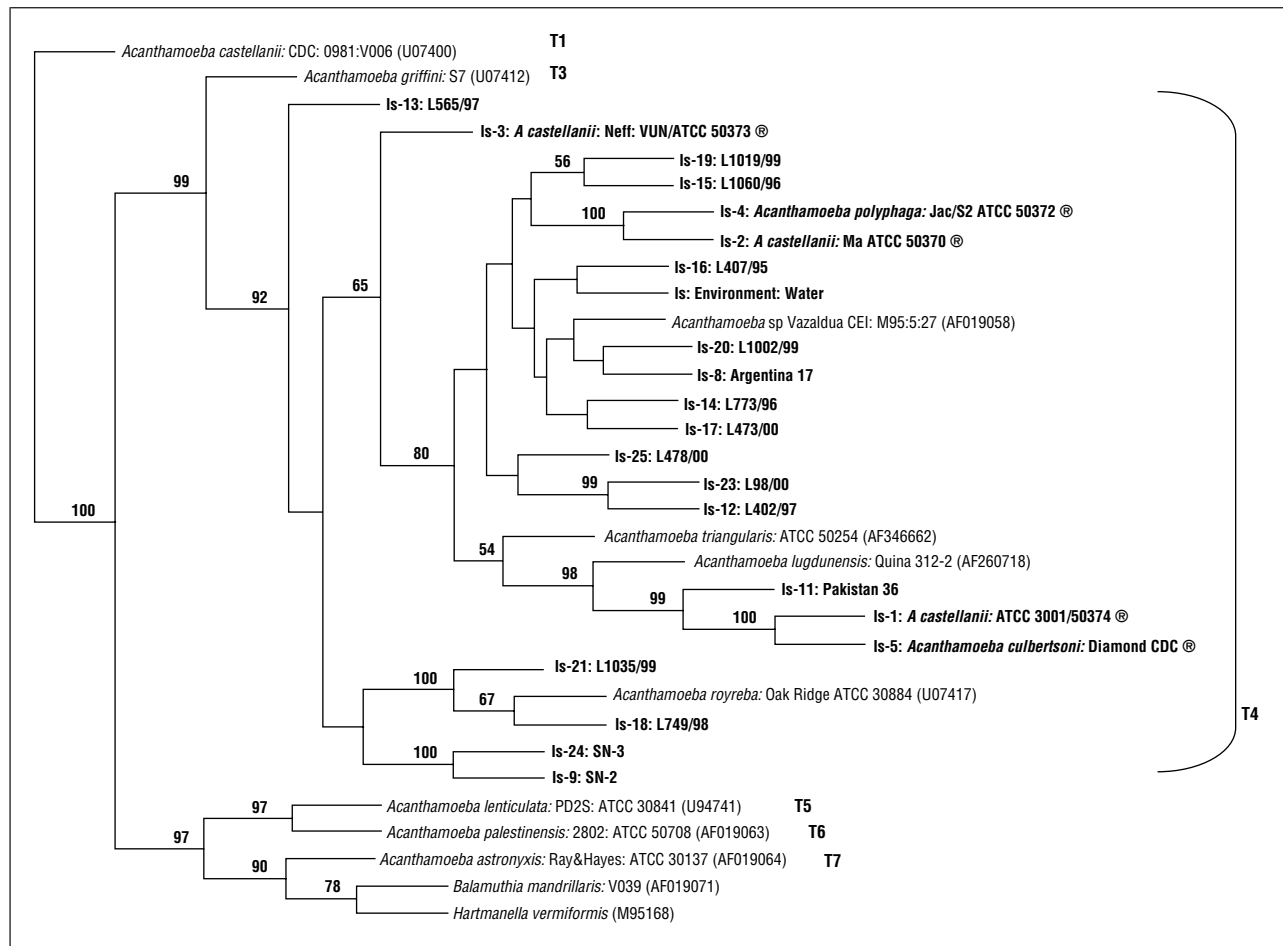
by very high bootstrap values that indicated the robustness and reliability of the rDNA sequence data obtained in the study for establishing the genetic affiliation of the analyzed amoeba isolates. Within themselves, the Indian isolates showed considerable variation, suggesting multiple *Acanthamoeba* species, as was also supported by their detailed morphometric analysis (data not shown). These results are in conformity with the original work of Gast et al,<sup>7</sup> which described the rDNA sequence types and showed that the T4 type sequence characterizes a heterogeneous group of pathogenic isolates of *Acanthamoeba* comprising many different species.

The attempts to develop a reliable diagnostic test led to the multiplex PCR assay having high sensitivity (5 pg of *Acanthamoeba* DNA) and specificity for the agarose gel-based detection of *Acanthamoeba* in clinical samples (Figure 2). The assay, being based on 2 markers (18S and 26S rDNA), provides relatively more reliable detection than the one described earlier based on only 18S rDNA<sup>8,12</sup> and also overcomes the limitations of using different amplification conditions for sensitivity and specificity.<sup>8</sup> The 18S rDNA primers that amplify a 463-bp amplicon from *Acanthamoeba* also produced a similar-sized amplicon for *Balamuthia* and *Hartmannella*, the 2 closely related protozoa, under the conditions used in our studies. However, in the multiplex assay (Figure 2) wherein primers specific to both 18S and 26S rDNAs were used together, the 463-bp fragment was not amplified from the related protozoa and only the 126-bp fragment was seen for *Balamuthia*. In contrast, a double-band pattern comprising 463 and 126 bp was obtained only with *Acanthamoeba*. The *Acanthamoeba*-specific 2-fragment phenotype may not indicate the presence of *Balamuthia* in the test sample if the latter is also present as an additional protozoan, although keratitis cases having such mixed protozoa infections are yet to be defined. Experiments done to test the diagnostic utility of the assay, using 34 clinical corneal scraping samples and DNA from 22 *Acanthamoeba* isolates and many nontarget organisms as negative controls (see earlier), resulted in only 1 false-negative finding, establishing the high specificity (>98%) of the assay. On the other hand, the 2-fragment phenotype on the agarose gel specifying *Acanthamoeba* was seen for 24 of the 25 culture-proved, *Acanthamoeba*-positive corneal scraping samples, showing high accuracy of positive identification (96%), and for none of the 9 corneal scrapings of patients with bacterial or fungal keratitis.

Perusal of the clinical data of the patients from whom the study samples were drawn did not suggest any bias with respect to the sex and age of the patients (Table) and the incidence of *Acanthamoeba* keratitis. Notably, all the patients belonged to relatively poor strata of society, none wore contact lenses, and for the few cases with known history, mechanical injury or trauma was found to be the main predisposing factor.

## COMMENT

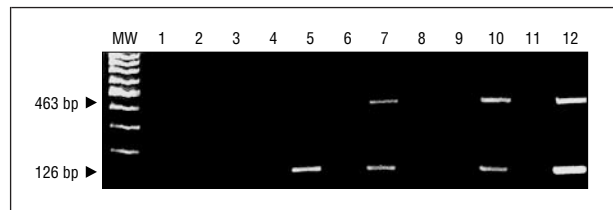
Results presented herein conclusively prove that *Acanthamoeba* is one of the causative organisms of keratitis in non-contact lens wearers from India. Ribosomal DNA



**Figure 1.** Phylogenetic tree determined by unweighted pair group method using arithmetic averages based on 18S ribosomal DNA typing showing genetic relationships of the putative *Acanthamoeba* isolates obtained from patients with keratitis, reference *Acanthamoeba* species, and related protozoa *Balamuthia* and *Hartmannella*. Note that all Indian isolates (entries labeled with prefixes Is-9, Is-12 to Is-21, Is-23 to Is-25, and Is-Environment) are included in the T4 cluster of reference acanthamoebae.<sup>7,11</sup> The 22 isolates analyzed in the study are printed in boldface. The entries marked with ® are the reference *Acanthamoeba* species sequenced in the present study. The numbers at the nodes are the bootstrap values. The accession numbers of the reference sequences obtained from the EMBL (European Molecular Biology Laboratory) database are given in parentheses. ATCC indicates American type culture collection.

typing-based genetic identity analysis showed a high degree of genetic diversity among the *Acanthamoeba* isolates (Figure 1), and all of these carried the T4 signatures that define the pathogenic forms.<sup>7</sup> These results emphasize that ophthalmologists and general physicians in India and other developing countries should be aware of *Acanthamoeba* as one of the causes of keratitis in non-contact lens wearers, and thus should take immediate measures for its early diagnosis and management. For the latter purpose, the simple PCR-based test described herein holds great promise, especially after such technology has become widely available in most parts of India and elsewhere. Even creation of a new setup for PCR-based testing is much easier and cost- and time-effective than the other relatively reliable approaches of fluorescence microscopy using calcofluor white staining<sup>5</sup> and culture-based methods.

Awareness about the disease and availability of an easy detection tool would not only help in early detection and better management of *Acanthamoeba* keratitis, a condition that presently largely goes undetected and uncured at most centers, but would also help greatly in obtaining realistic estimates of disease incidence. Thus,



**Figure 2.** Representative gel showing ribosomal DNA typing of different DNA samples by means of multiplex polymerase chain reaction assay for direct detection of *Acanthamoeba*. DNA in lane 1, negative control; 2, bacterial (*Pseudomonas aeruginosa*); 3, viral (herpes simplex virus); 4, fungal (*Aspergillus* species); 5, *Balamuthia*; 6, *Hartmannella*; 7 to 10, corneal scrapings of patients with keratitis culture proven to be *Acanthamoeba* (7, 10), bacterial (8), or fungal (9); 11, human; and 12, positive control *Acanthamoeba castellanii*. Note amplicons of approximately 463 and 126 base pairs (bp) only in *Acanthamoeba* lanes 7, 10, and 12, and 126 bp only in *Balamuthia* lane 5. MW indicates molecular weight.

the diagnostic and epidemiologic value of the multiplex PCR cannot be overemphasized for countries like India where such data are lacking.

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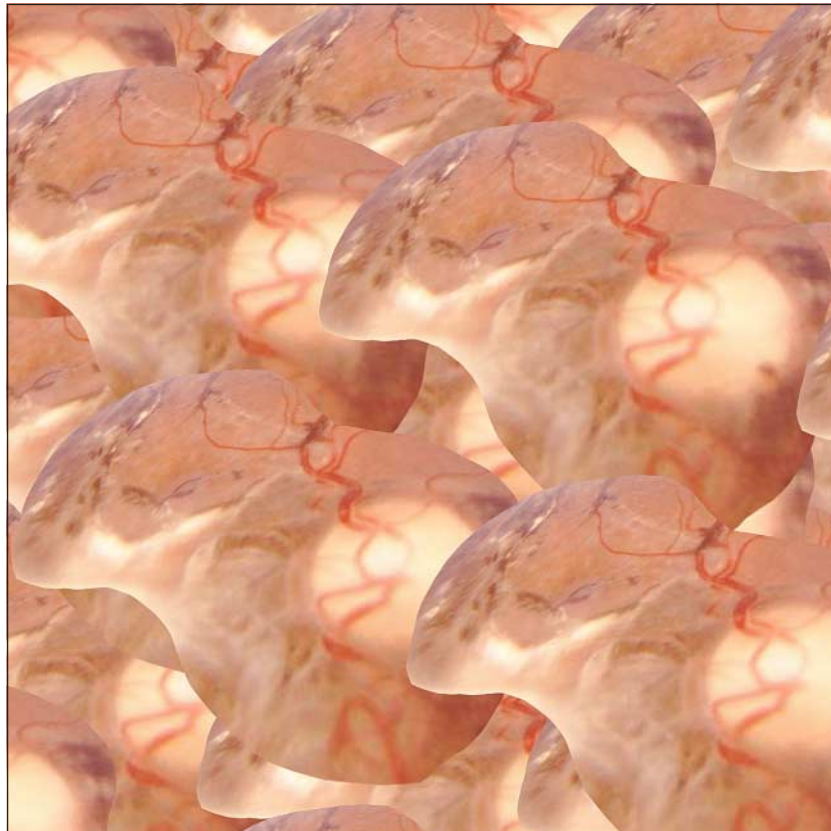
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